# REGULATED EXPRESSION OF PKC AND/OR SRB1/PSA1 IN YEAST

The present invention relates to genetically modified yeasts in which the expression of genes responsible for cell wall synthesis are modulated.

The yeast cell wall is a dynamic organelle responsible for a number of cellular functions, the most important being physical and osmotic protection, selective permeability, cell-cell recognition and adhesion during mating and flocculation.

The cell wall of the yeast *Saccharomyces cerevisiae* is composed of three components, β-glucan (a glucose polymer), mannoproteins and chitin (an N-acetylglucosamine polymer). The β-glucan component of the cell wall consists of two polymers: a large, linear β-1,3-glucan and a smaller, highly branched β-1,6-glucan moiety whereas the mannoproteins are a complex of proteins modified by the attachment, via N- and O-glycosidic bonds, of mannose-containing carbohydrate chains of different length and structure.

The yeast cell wall is a very rigid, highly complex structure which determines the shape of the yeast cell and enables it to be protected from and adjusted to its ever-changing environment. A growing number of genes have been shown to participate in the biosynthesis and assembly of the major cell wall components, some of them as part of well-defined signal transduction pathways. For instance, *PKC1* (the yeast homologue of protein kinase C) regulates the biosynthesis and assembly of major cell wall components by a *PKC1*-mediated signal transduction pathway. *PKC1*. in conjunction with Rho1p, regulates β 1-3 glucan synthetase Null mutants of *PKC1* can only grow in the presence of osmotic stabilisers. Loss of *PKC1* function results in a cell-cycle-specific osmotic stability defect.

A further gene relevant to the cell wall is the recently identified *SRB1* (also known as *PSA1* or *VIG9*) yeast gene which is essential for growth and encodes GDP-

mannose pyrophosphorylase, an enzyme responsible for the production of a major substrate for all kinds of mannosylation reactions, including the biosynthesis of cell wall mannoprotein. A *SRB1/PSA1* null mutation is lethal whereas a decrease in *SRB1/PSA1* function (by inhibiting the expression of *SRB1/PSA1)* leads to defects in bud growth, bud site selection, and cell separation, in addition to increases in cell permeability and cell lysis.

Another gene which regulates the cell wall is *PDE2* (the gene encoding a high-affinity cAMP phosphodiesterase) which is part of the RAS/cAMP dependent pathway in yeast. We recently identified *PDE2* as a multi-copy suppressor of srb1-1 (a mutation which depends on sorbitol for growth). Moreover, strains carrying a *pde2* deletion share a number of phenotypes with *srb1-1* mutants, including lysis upon osmotic shock.

The yeast cell wall influences the characteristics of the yeast cell in a number of ways and it is desirable to modulate the wall to confer desirable properties on yeast.

For instance, the yeast cell wall acts as a barrier which can obstruct the liberation of protein expressed within yeast cells. This effect is of particular significance to the biotechnology industry which uses yeasts for protein production and for the production of recombinant proteins in particular. For some proteins, it is possible to use the yeast secretion pathway to release the protein from the cell, thus obviating the need for mechanical or enzymic degradation of the cell wall. However, many proteins cannot be exported by the secretion pathway and are retained within the cell in a membrane-associated form. Some protein complexes, such as virus-like particles (VLPs) are also impossible to recover by the secretion route. It may be seen, therefore, that the rigid cell wall of yeast is a major barrier to the efficient operation of downstream processes leading to protein isolation and purification. In the food industry, yeast cell extracts are used as dietary supplements and flavourings, again

requiring efficient methods of cell lysis that do not compromise the nutritional or organoleptic properties of the yeast cell extract.

The use of lysis mutants represent an alternative to mechanical/chemical disruption for the efficient recovery of yeast cell contents. Some attempts have been made to develop such mutants. For instance, WO 92/01798 concerns the use of a *srb1-1* mutant. However this specification related to a DNA sequence deposited under the Budapest treaty which was first believed to code for the *SRB1* gene but has since been shown to be the *PDE2* gene. WO 96/02629 also relates to a lysis mutant and concerns the use of a *mpk1/slt2* mutant which is stated to release intracellular proteins, including VLPs. following a temperature shift and osmotic shock. However, the application of both types of lytic mutants has significant drawbacks. The osmotic stabiliser required to enable the growth of a *srb1-1* mutant is either expensive (sorbitol) or corrosive to the fermentor (NaCl) thus precluding the use of this mutant in large-scale processes. The temperature shift involved in the use of the *mpk1/slt2* mutant requires considerable energy input and can also trigger the degradation or re-modelling of the proteins released from the lysed cells.

It is therefore an object of the present invention to provide yeast mutants which obviate or mitigate the abovementioned disadvantages.

According to a first aspect of the invention, there is provided a yeast cell containing the *SRB1/PSA1* gene and the *PKC1* gene or functional derivatives thereof each operatively linked to a heterologous inducable promoter.

We have found that yeast cells containing the *SRB1/PSA1* gene and the *PKC1* gene or functional derivatives thereof each operatively linked to an inducable promoter (e.g. the cells according to the first aspect of the invention) may be used in applications where the induction of cell lysis is desirable. For instance, induction of yeast cell lysis is useful for isolating protein expressed within a yeast cell which is not

readily secreted into the medium in which the cells are growing. Thus according to a second aspect of the present invention, there is provided a method of regulating yeast cell lysis comprising:

(i) growing yeast cells containing the *SRB1/PSA1* gene and the *PKC1* gene or functional derivatives thereof each operatively linked to an inducable promoter in a growth medium which activates the inducable promoter such that *SRB1/PSA1* and *PKC1* are expressed from said cells; and

(ii) when lysis is required, growing the cells in a modified growth medium which represses *SRB1/PSA1* and *PKC1* expression such that cell lysis is induced.

The present invention is based upon our efforts to develop conditional lysis mutants that do not require special media in which to grow (e.g. sorbitol supplemented) or temperature shifts. We placed various genes that have been shown to contribute, in different ways, to cellular integrity of *S. cerevisiae* under the control of inducable promoters and examined whether or not repression of the gene (by altering the composition of the media in which the yeast is grown such that the promoter is inactivated) modulates cell lysis. We found that repression of some of the genes tested, for instance *PDE2*, did not significantly influence lysis. These genes were therefore unsuitable candidates to be modulated to generate an improved lytic yeast strain.

We found that repression of the *SRB1/PSA1* gene in yeast cells grown in normal media resulted in a reduction in cell growth, cells gradually losing their viability and integrity and the release of about 7% of total protein into the medium 24 hours after repression was induced. Furthermore we found that repression of *PKC1* led to more extensive release of cellular protein into the medium (approximately 18% of total protein into the medium 24 hours after repression was induced) although cell growth was not significantly altered. These results were expected because, although it

is known that yeast cells carrying the *srb1-1* allele can grow in osmotically-buffered media, such mutants lyse upon hypoosmotic shock. It is also known that cells bearing the *pkc1* mutation can grow in osmotically-bufferred media (e.g. in the presence of 10% (w/v) sorbitol) but quickly lyse upon osmotic shock.

We also repressed expression of both *SRB1/PSA1* and *PKC1* in yeast cells and found that under these circumstances the yeast cultures underwent substantial lysis which permitted the efficient release of both homologous and heterologous proteins from the yeast. In fact, as illustrated in Example 1, lysis was surprisingly more extensive than observed for cells in which *SRB1/PSA1* and *PKC1* were repressed singularly (about 30% of total protein was released into the medium 24 hours after repression was induced) which shows that the lysis phenotype conferred by the repression of *SRB1/PSA1* and *PKC1* was additive. Thus cells according to the first aspect of the invention are of particular utility as it is possible to grow the cells without significant lysis and then at a predetermined time during the fermentation induce extensive lysis of the yeast cells.

Cells according to the first aspect of the invention may be formed from yeast strains with normal *SRB1/PSA1* and *PKC1* expression. Preferably the yeast is *Saccharomyces cerevisiae* or strains thereof. Examples of such yeast strains include ZO123 and FY23. The conservation of gene function in different yeast species means that other types of yeasts (particularly those that are currently exploited for heterologous gene expression such as *Pichia pastoris, Hansenula polymorpha* and *Kluyveromyces lactis)* may be used to form cells according to the first aspect of the invention in which their *SRB1/PSA1* and *PKC1* homologues are operatively linked to an inducable promoter.

The endogenous promoters of the *SRB1/PSA1* gene and the *PKC1* gene are not readily inducable in such strains and it is therefore necessary to modify genetically yeast such that *SRB1/PSA1* and *PKC1* expression from the yeasts is inducable. This

may be achieved in a number of ways. For instance, yeast cells may be transformed with DNA molecule(s) comprising an inducable promoter(s) such that the inducable promoter(s) take over control of transcription of the endogenous *SRB1/PSA1* and *PKC1* genes. These DNA molecules are preferably designed such that they will integrate into the yeast genome and replace the region of DNA containing the endogenous *SRB1/PSA1* and *PKC1* promoters (as appropriate). As a result the inducable promoter introduced into the cell becomes operatively linked to the *SRB1/PSA1* and *PKC1* genes and can control their expression. Alternatively the cells may be transformed with a first recombinant DNA molecule comprising an inducable promoter operatively linked to the *SRB1/PSA1* gene and/or a second recombinant DNA molecule comprising an inducable promoter operatively linked to the *PKC1* gene. These recombinant DNA molecules are designed such that they will integrate and replace by homologous recombination the endogenous *SRB1/PSA1* and *PKC1* genes respectively. The DNA molecules and recombinant DNA molecules used for transforming yeast cells are preferably incorporated in a suitable vector which bears a DNA sequence which allows homologous recombination between the vector and the DNA at the site of the endogenous promoter / gene.

The cells according to the first aspect of the invention may also be derived from yeasts which are *srb1-1* and/or *pkc* mutants. These mutants have a lytic phenotype and are only able to survive when grown in osmotically buffered media. However we have found that these cells may be transformed with an expression cassette comprising an inducable promoter and DNA sequences encoding suitable genes to replace the mutated gene to form cells according to the first aspect of the invention which display a normal phenotype (i.e. they are not osmotically sensitive or liable to lyse spontaneously) in permissive growth media conditions (which allows activation of the inducable promoter) but will lyse when the media is modified such that gene expression is repressed. Yeast cells to be modified may be transformed with the abovementioned recombinant DNA molecules (or vectors bearing such molecules) to form cells according to the first aspect of the invention in which the recombinant

DNA molecules either integrate into the genome of the mutant yeast or which may subsist (and ideally autonomously replicate) in the cytosol of the yeast cell. Examples of *srb1-1* and/or *pkc* mutant cells which may be used include the ZO124 strain of *Saccharomyces cerevisiae.*

The *SRB1/PSA1* gene and the *PKC1* gene (or functional derivatives thereof ) may each be operatively linked to a number of inducable promoters. The inducable promoter may. for example, be the *GAL1* promoter (inducable by galactose) or the *TET* promoter (inducable by tetracyclin).

Preferred promoters are ones which may be regulated by an agent contained within the media within which the cells are grown. Such agents are ideally readily available, inexpensive, soluble in normal yeast growth medium and do not adversely effect proteins released from lysed cells. We have found that the methionine regulated promoter, p*MET3* (Mountain *et al.*, 1991 Yeast 7: 781 - 803) fulfils these criteria as its modulator (methionine) may be easily included or excluded in growth medium as required. Thus *pMET3* is a preferred promoter.

The p*MET3* promoter drives gene expression in the absence of methionine. Therefore in methionine-free media *SRB1/PSA1* and *PKC1* expression occurs. However when the media is modified by the addition of methionine, gene expression is repressed and cell lysis induced.

DNA sequences corresponding to p*MET3* may be used as a DNA molecule for transforming yeast cells although it is preferred that p*MET3* is contained within a vector (i.e. as part of a larger DNA molecule containing other functional elements). A preferred vector. named pRS316-p*MET3*, comprises a pRS316-based plasmid (described in Sikorski & Heiter (1989) Genetics 122: 19-27) which contains the *MET3* promoter (Yeast genome Accession no. X06413). The construction of pRS316-p*MET3* is described in detail in Example 1.

pRS316-p*MET3* may be used to form recombinant vectors which contain preferred recombinant DNA molecules p*MET3-PKC1* and p*MET3-SRB1*.

Preferred derivatives of pRS316-p*MET3* which contain p*MET3-PKC1* include pRS316-p*MET3-PKC1* is described in detail in Example 1. Other preferred derivatives of pRS316-p*MET3* are designed to allow integration of the p*MET3-PKC1* regulation cassette at the homologous *PKC1* locus. For instance, pRS316-$F_1F_2$-p*MET3-PKC1* is constructed by inserting a *PKC1* upstream flanking region (which was designated $F_1F_2$ and has the nucleotide sequence listed below) between the *Kpn*I and *Sph*I sites of pRS316-p*MET3-PKC1*.

Nucleotide sequence of the $F_1F_2$ DNA fragment:

ACAAGCAGCTGATGAAAAGCCAAGACATAAGTATTGT TGCCCACACT
GTGGGTCTTCATTTCCAAGATGTGCCATATGTCTCATGCCTCTAGGAA
CGTCAAACTTACCTTTTGTAATAAATGGGACGCAATCACGCGATCAAT
GCAGACAGAAGACTCTCAAGATGGTGCAAATCGCGAACTCGTAAGTA
GAAAACTGAAGTTGAACGAGTGGTTCAGCTTCTGTTTGAGTTGCAACCA
TGGTATGCATGCCGGTCACGCTGAAGAATGGTTTGACAGACATAATGTT
TGTCCCACTCCAGGTT

(SEQ. I.D. NO. 14)

pRS316-$F_1F_2$-p*MET3-PKC1* may also be further modified to introduce the *TRP1* gene as a selectable marker (a DNA molecule corresponding to Yeast genome accession No. V01341 or J01374) between the *Sph*I and *Sal*I sites to form the construct pRS316-$F_1F_2$-*TRP1*-p*MET3-PKC1*. pRS316-$F_1F_2$-*TRP1*-p*MET3-PKC1* is particularly useful when forming cells according to the first aspect of the invention because it may be digested with *Kpn*I and *Sac*I and the fragment containing $F_1F_2$-*TRP1*-p*MET3-PKC1* used to transform a host yeast strain.

SRB1.9e is a preferred recombinant vector which contains the recombinant DNA molecule p*MET3-SRB1* and is described in more detail in Example 1. Plasmid

pSRB1-9e is particularly useful when forming cells according to the first aspect of the invention because it may be digested with *Apa*l and *Bst*1107I and the p*MET3-SRB1-LEU2* fragment obtained in this way used to transform a yeast cell.

Preferred cells according to the first aspect of the invention may be formed using the above described constructs. For instance, yeast strain ZO-123 (*MATa his3 leu2 trp1 ura3)* which expresses both *SRB1/PSA1* and *PKC1* may be modified such that the transcription of the endogenous *SRB1/PSA1* and *PKC1* genes are brought under the control of p*MET3* (e.g. by integrating p*MET3* into the yeast genome such that it replaces the endogenous *SRB1* and *PKC1* promoters). Alternatively pRS316-$F_1F_2$-p*MET3-PKC1* or pRS316-$F_1F_2$-TRP1-p*MET3-PKC1* in conjunction with SRB1.9e may be used to integrate the *pMET3-PKC1* and *pMET3-SRB1* cassettes respectively into ZO-123 to form ZO-127 which is a particularly preferred cell according to the first aspect of the invention.

We have found that lysis may be regulated according to the method of the second aspect of the invention by growing yeast cells according to the first aspect of the invention in a growth medium which activates the inducable promoter such that *SRB1/PSA1* and *PKC1* are expressed from said cells. Then, after a predetermined time. the cells may be switched to growth in the modified growth medium such that *SRB1/PSA1* and *PKC1* expression is repressed and cell lysis induced.

The manner in which *SRB1/PSA1* and *PKC1* gene expression is regulated according to the method of the second aspect of the invention will depend upon which inducable promoter is being used. This regulation is dependent upon the exact concentration of an agent capable of modulating promoter activity contained within the growth medium. We have found that addition to the media of methionine to a concentration of between 0.05mM and 20mM will inhibit expression of *SRB1/PSA1* and *PKC1* from cells transformed with p*MET3-SRB1* and p*MET3-PKC* and thereby induce lysis whereas the same cells grown in the absence (or minimal concentration)

of methionine are able to grow unimpeded. Preferably a concentration of between about 0.05mM and 5mM methionine in the media and most preferably a concentration of about 2mM methionine in the media is used to induce lysis.

The growth medium used according to the method of the second aspect of the invention should be readily adaptable such that it may be in either of two forms: one which permits activation of the inducable promoter and thereby *SRB1/PSA1* and *PKC1* expression; and a second form which is modified such that *SRB1/PSA1* and *PKC1* gene expression is repressed. This repression may be effected by removal of an agent which activates the promoter but is preferably effected by addition to the media of an agent which inhibits the promoter.

The growth medium should contain sufficient amounts of nutrients (i.e carbohydrate, nitrogen source etc) required to allow optimal growth of yeast when *SRB1/PSA1* and *PKC1* are not being repressed.

The exact composition of the medium depends upon a number of factors (for instance the specific yeast used). Purely by way of example a suitable growth medium is F1 medium which comprises:

| | final concentration in F1-medium |
|---|---|
| <u>Mineral salts</u> | |
| Ammonium sulphate | 3.13 g/l |
| Potassium dihydrogen orthophosphate | 2.00 g/l |
| Magnesium sulphate 7-hydrate | 0.55g/l |
| Sodium chloride | 0.10g/l |
| Calcium chloride dihydrate | 0.09g/l |
| <u>Trace elements</u> | |
| Zinc sulphate 7-hydrate | 0.07mg/l |
| Ferric chloride 6-hydrate | 0.05mg/l |
| Cupric sulphate | 0.01mg/l |
| Boric acid | 0.01mg/l |
| Potassium iodide | 0.01mg/l |
| <u>Vitamins</u> | |
| Inositol | 62.00mg/l |
| Thiamine Hydrochloride | 14.00mg/l |

| | |
|---|---|
| Pyridoxine | 4.00mg/l |
| Calcium Pantothenate | 4.00mg/l |
| d-Biotin | 0.30mg/l |

+ carbohydrate substrate

+/- agent which modulates the inducable promoter

The composition of the media and the modified form thereof ideally only differ by the inclusion or exclusion of an agent which modulates the inducable promoter. The type of agent used will depend upon which specific promoter is used. When cells are used in which *SRB1/PSA1* and *PKC1* are operatively linked to the *pMET* promoter. the media permissive for yeast cell growth should be free of methionine. Methionine may be added to the medium as required to form the modified media in which lysis is induced.

The method of the second aspect of the invention may be readily adapted for the purposes of isolating protein from yeast cells. Once yeast cells have been lysed yeast cell debris / ghosts may easily be separated from the protein released from the cells (e.g. by filtration, sedimentation and/or centrifugation). The protein may then be further purified using conventional biochemical techniques. The method is most suitable for isolating recombinant proteins expressed from genetically engineered yeast cells.

Another characteristic of yeast which is determined by the cell wall is its ability to flocculate. Unlike adhesion in mating, which is induced by highly specific pheromones, flocculation is an asexual aggregation of cells which is a very useful characteristic in industrial yeast strains. Flocculation is exploited in fermentations such as beer-brewing, wine-making, and fuel ethanol production because it leads to efficient separation of cells from the fermentation liquor.

Two types of flocculation phenotypes have been described. The *FLO1* type, caused by *FLO1/FLO5/FLO8*, is $Ca^{2+}$-dependent and inhibited by mannopyranoses. The NewFlo phenotype, on the other hand, is prevented by both manno- and glucopyranoses. The *FLO1* gene, which is located on chromosome I, has been reported to encode a GPI-anchored, cell surface protein with its amino terminus exposed to the medium. *FLO5* is highly homologous to *FLO1* and is also found on chromosome I. *FLO8*, previously mapped to chromosome I and said to be allelic to *FLO1* has recently been reassigned to chromosome V and demonstrated to mediate flocculation via transcriptional activation of *FLO1*. More recently, a new flocculation gene, named *FLO2*, has been cloned and localised to chromosome XII; its function remains unclear, although it can complement *flo1* mutations. Other genes, like *TUP1* and *SNN6*, also act on yeast cell flocculation via transcriptional regulation.

Flocculation in *S. cerevisiae* is thought to be a result of interactions between lectin-like cell surface proteins (termed flocculins), encoded by the *FLO* genes, and the cell wall mannan. This hypothesis is supported by the following findings: loss of flocculation capacity following protease treatment, efficient dispersion of flocs by mannose and its derivatives and the failure of certain *mnn* mutant cells to co-flocculate with flocculant cells. So far, studies of flocculation have centred on the cloning and characterisation of dominant flocculation genes and the elucidation of their transcriptional regulation. Less attention has been paid to the effect of changes of cell wall structure on flocculation.

We have found that cells in which the *PKC1* and/or *SRB1/PSA1* gene or functional derivatives thereof are operatively linked to heterologous promoters may be used in applications in which the induction of flocculation is desirable (e.g. fermentation reactions such as for the production of alcohol).

According to a third aspect of the present invention, there is provided a method of regulating yeast cell flocculation comprising:

(i) growing yeast cells containing the *PKC1* gene or functional derivatives thereof operatively linked to an inducable promoter in a growth medium which activates the inducable promoter such that *PKC1* is expressed; and

(ii) when flocculation is required, growing the cells in a modified growth medium which represses *PKC1* expression such that flocculation is induced.

According to the third aspect of the invention we have found that the repression of *PKC1* expression makes it possible to induce flocculation. Therefore cells containing the *PKC1* gene or functional derivatives thereof operatively linked to a heterologous inducable promoter (such as p*MET3*) are useful when it is desired to induce flocculation. Such cells are particularly useful when the simultaneous induction of lysis and flocculation is required (as *PKC1* repression also causes lysis). The induction of lysis and flocculation is desirable when purifying proteins released from yeasts. The induced lysis liberates the contents of the cell whereas the induced flocculation will favour sedimentation of the cell ghosts / debris and thereby separate cell contents (which will remain in the media) from the cell ghosts / debris.

Cells suitable for use according to the method of the third aspect of the invention include cells in which only *PKC1* is under the regulation of an inducable promoter and include:

(i) ZO124 transformed with pRS316-p*MET3-PKC1*, pRS316-$F_1F_2$-p*MET3-PKC1* or pRS316-$F_1F_2$-TRP1-p*MET3-PKC1* (see Example 1);

(ii) ZO123 transformed with pRS316-p*MET3-PKC1* or p*MET3-PKC1* containing fragments derived from pRS316-$F_1F_2$-p*MET3-PKC1* or pRS316-$F_1F_2$-TRP1-p*MET3-PKC1* (see Example 1); and

(iii) yeast strain ZO-126 (see Example 2).

We have also found that cells may be developed which have a flocculating phenotype by removing *SRB1/PSA1* from under the control of its endogenous promoter and placing the gene under the control of a heterologous promoter (which may be inducable or constitutive). Such cells flocculate but do not lyse to a significant extent and are therefore useful in industrial applications where flocculation is of primary importance (e.g. for sedimenting yeast during the brewing process). Thus according to a fourth aspect of the invention there is provided a method of fermentation comprising growing yeast cells containing the *SRB1/PSA1* gene or functional derivatives thereof operatively linked to a heterologous promoter in a growth medium in which *SRB1/PSA1* expression is regulated by the heterologous promoter whereby said cells flocculate.

*SRB1/PSA1* expression may be regulated in cells used according to the fourth aspect of the invention by an inducable promoter or a constitutive promoter. p*MET3* is a preferred promoter for regulating *SRB1/PSA1* expression in cells used according to the fourth aspect of the invention.

Examples of cells in which *SRB1/PSA1* is under the regulation of an inducable promoter include:

(i) ZO125 (ZO 123 cells transformed with p*MET3-SRB1*); and

(ii) FY23*SRB1*$^{MET3}$.

Although we do not wish to be bound by any hypothesis we believe that the flocculation phenotype caused when *SRB1/PSA1* is transcribed from *pMET3* is not due to the gene's underexpression but, rather, is the result of its constitutive expression. Cell viability is not affected by the constitutive expression of *SRB1/PSA1*, suggesting that sufficient Srb1/Psa1p is synthesised under the control of *pMET3* to allow yeast to go through its cell cycle. However, when *SRB1/PSA1* is expressed from its own promoter, its transcription level increases some 4- to 6-fold at START. Thus the constitutive expression of *SRB1/PSA1* from *pMET3* could hyperactivate

glycosylation at all other cell cycle phase which may lead to enhanced cell growth and flocculation.

According to a fifth aspect of the invention there is provided a method of fermentation comprising growing yeast cells containing the *SRB1/PSA1* and *PKC1* gene or functional derivatives thereof operatively linked to a heterologous promoter in a growth medium in which *SRB1/PSA1* and *PKC1* expression is regulated by the heterologous promoter whereby said cells flocculate.

We believe cells used according to the method of the fifth aspect of the invention have a flocculating phenotype because *SRB1/PSA1* is not regulated by its endogenous promoter in such cells. These cells may comprise:

(i) *PKC1* operatively linked to an inducable promoter and *SRB1/PSA1* linked to any heterologous promoter; or

(ii) both *PKC1* and *SRB1/PSA1* operatively linked to an inducable promoter (i.e. cells according to the first aspect of the invention).

The method of the fifth aspect of the invention may be used when it is desirable to induce lysis (e.g. according to the method of the second aspect of the invention) at a predetermined time during the fermentation as well as flocculation. For instance, this may be achieved by adding methionine (0.05mM - 20 mM) to the growth medium when *PKC1* is operatively linked to a methionine regulated promoter such as *pMET3*.

According to a sixth aspect of the invention, there is provided a yeast cell containing the *PKC1* gene or functional derivatives thereof operatively linked to a heterologous inducable promoter.

Cells according to the sixth aspect of the invention may be employed in the method according to the third aspect of the invention. Such cells may contain the

*PKC1* gene or functional derivatives thereof operatively linked to any inducable promoter described above for use in cells according to the first aspect of the invention.

Preferred cells according to the sixth aspect of the invention include:

(i) ZO124 transformed with pRS316-p*MET3-PKC1*, pRS316-$F_1F_2$-p*MET3-PKC1* or pRS316-$F_1F_2$-TRP1-p*MET3-PKC1* (see Example 1);

(ii) ZO123 transformed with pRS316-p*MET3-PKC1* or p*MET3-PKC1* containing fragments derived from pRS316-$F_1F_2$-p*MET3-PKC1* or pRS316-$F_1F_2$-TRP1-p*MET3-PKC1* (see Example 1); and

(iii) yeast strain ZO-126 (see Example 2).

According to a seventh aspect of the invention, there is provided a yeast cell containing the *SRB1/PSA1* gene or functional derivatives thereof operatively linked to a heterologous promoter.

Cells according to the seventh aspect of the invention may be employed in the method according to the fourth aspect of the invention. Such cells may contain the *SRB1/PSA1* gene or functional derivatives thereof operatively linked to any heterologous promoter (including inducable promoters). Preferred promoters are described above for use in cells according to the first aspect of the invention.

Examples of cells according to the seventh aspect of the invention include:

(i) ZO125 (ZO 123 cells transformed with p*MET3-SRB1*); and

(ii) FY23*SRB1*$^{MET3}$.

According to a eighth aspect of the invention, there is provided a yeast cell containing the *PKC1* gene or a functional derivative thereof operatively linked to a heterologous inducable promoter and the *SRB1/PSA1* gene or a functional derivative thereof operatively linked to a heterologous promoter.

Cells according to the eighth aspect of the invention may be employed according to the method of the fifth aspect of the invention. Such cells may be the same as cells according to the first aspect of the invention except the *SRB1/PSA1* gene or a functional derivative thereof may be operatively linked to any heterologous promoter.

Optimal growth of yeasts used according to either the methods of the second or third aspects of the invention can be dependent upon the fermenter in which the yeast are grown. Fermenters will usually comprise one or more of:

1. Rotors or similar devices for agitating the yeast culture.
2. An air (or oxygen) supply.
3. An inlet for addition of nutrients or agents which modify the medium.
4. A means of extracting waste products and / or proteins produced
5. A thermostat and means of regulating temperature.

Preferred fermenters are those already known to the art for the culture of yeast. The type of fermenter used will depend upon whether the yeast cells are being grown in the laboratory. by potage, as a pilot plant or in full industrial scale-up (e.g. for industrial production of yeast proteins).

When cells are cultured in the modified growth medium (which represses *SRB1/PSA1* and *PKC1* expression such that lysis is induced) according to the second aspect of the invention, the culture conditions do not need be as stringently regulated as during the growth phase because cell viability is not relevant when lysis is induced. However it will be appreciated that the media should not be allowed to change (e.g. undesirable pH or temperature changes) such that the liberated yeast cell contents (e.g. a recombinant protein) are denatured or corrupted.

The culture conditions required for cells grown in modified growth medium according to the third aspect of the invention will depend upon whether flocculation only (whilst maintaining cell viability) or whether flocculation and lysis is desired. If it is desired to maintain viability similar culture conditions as used for growth in the permissive media should be maintained whereas if lysis is to be induced the comments of the preceding paragraph apply.

The present invention will now be described, by way of example, with reference to the accompanying drawings in which:

Figure 1 schematically represents the DNA sequences inserted into the pRS316 vector in the construction of pRS316-pMET3 (a), pRS316-pMET3-PKC1 (b) and pRS316-$F_1F_2$pMET3-PKC1 (b);

Figure 2. is a graph illustrating the effect of *SRB1* repression on cell viability and cell lysis in Example 1;

Figure 3. is a graph illustrating the effect of *PKC1* repression on cell viability and cell lysis in Example 1;

Figure 4. is a graph illustrating the effect of dual *SRB1* and *PKC1* repression on cell viability and cell lysis in Example 1;

Figure 5. is a bar chart illustrating the GFP released from *SRB1* and *PKC1* repressed cells (ZO127/Pope-2μ) in Example 1;

Figure 6 is a photograph illustrating Flocs of ZO126 under expression conditions ($Met^-$, no methionine) and repression conditions ($Met^+$, 2mM methionine) in Example 2;

Figure 7 is a bar chart illustrating the percentage of Z0123 and ZO126 cells flocculated in the absence or presence of methionine in Example 2;

Figure 8 is a photograph illustrating Flocs of ZO123 (a), ZO125 (b) and ZO123/ SRB1.9e (c) grown under expression conditions ($Met^-$, no methionine) in Example 2;

Figure 9 is a bar chart illustrating the percentage of Z0123, ZO125 and ZO123/ SRB1.9c cells flocculated in the absence or presence of methionine in Example 2;

Figure 10 is a photograph illustrating Flocs of ZO127 grown under expression conditions ($Met^-$, no methionine) or repression ($Met^+$, 2mM methionine) in Example 2; and

Figure 11 is a bar chart illustrating the percentage of Z0123 and ZO127 cells flocculated in the absence or presence of methionine in Example 2.

**EXAMPLE 1**

Yeast according to the first aspect of the invention were made by transforming suitable yeast strains and the extent of inducable cell lysis assessed relative to single *SRB1* and *PKC1* mutants.

## 1.1 Materials and Methods

### 1.1.1 Stains

Strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1 together with relevant references and sources.

Table1— *S.cerevisiae* strains used in this study

| Yeast strains | Relevant phenotype |
|---|---|
| FY23 | *MATa ura3-52 leu2-Δ1 trp1-Δ63* |
| FY23-SRB1*MET3* | *MATa ura3-52 leu2-Δ1 trp1-Δ1 psa1Δ::* p*MET3- SRB1-LEU2* |
| ZO123 | *MATa his3 leu2 trp1 ura3* |
| ZO124 | *MATa his3 leu2 trp1 ura3 pkc1::LEU2* |
| ZO125 | *MATa his3 leu2 trp1 ura3 psa1Δ:: pMET3- SRB1-LEU2* |
| ZO126 | *MATa his3 leu2 trp1 ura3 pkc1Δ::TRP1-pMET3-PKC1* |
| ZO127 | *MATa his3 leu2 trp1 ura3 pkc1Δ::TRP1-pMET3-PKC1 psa1Δ:: pMET3- SRB1-LEU2* |

Bacterial strains (JM109 and XL1-Blue, Premega) were grown in LB and LB + ampicillin, prepared as described by Sambrook *et al.* (Molecular Cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 1989).

### 1.1.2 Media

Yeast growth media (YEPD, minimal,) were prepared as described by Sherman *et al.* (Methods in yeast genetics. Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York, 1986) and Kaiser *et al.* (Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994). All media used for the lysis test were supplemented with 10% w/v sorbitol.

Selective drop-out media (modified growth medium) with appropriate combinations of bases and amino acids (e.g. +/- methionine)

### 1.1.3 Oligonucleotides and PCR

The sequences of the oligonucleotides employed in this study, and their utility, are given in Table 2. All PCR reactions were performed using *pwo* DNA polymerase (Boehringer Mannheim) according to the recommendation of the manufacturer. PCR products were purified (using a Qiagen kit, cat no. 28036) to eliminate primer dimers, salts. oligonucleotides and enzymes prior to restriction digestion .

### 1.1.4 Plasmids, DNA preparation and manipulations.

The plasmids employed in this study are listed in Table 3. Bacterial plasmid DNA was isolated using the alkaline-lysis method of Birnboim and Doly (*Nucl. Acids Res.* **7:** 1513-1523, 1979) as described by Sambrook *et al.* (*supra*). All DNA fragments used for sub-cloning were either PCR amplified and purified using a Qiagen kit (Cat. no. 28036) or gel-purified by the method of Heery *et al.* (*Trends Genet.* **6:** 173, 1990) after appropriate restriction enzyme digests. Enzymatic reactions with restriction enzymes, T4 DNA ligase, calf intestinal phosphatase and T4 DNA polymerase, were performed according to the recommendations of the manufacturers. Yeast genomic DNA was isolated from 5ml of yeast culture according to the method of Kaiser *et al.* (Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1991)

Table 2—Oligonucleotides used in the study

| SEQ ID No | Sequence | Description | Incorporation Site |
|---|---|---|---|
| 1 | GAAA **GCGGCCGC** AAA AGT AAG TAT AGT ATC AC (SEQ ID NO:1) | *PKC1* forward primer | *Not*I |
| 2 | CCT **GAGCTC** CAA GAT AGG TAC GAA CAA AA (SEQ ID NO: 2) | *PKC1* reverse primer | *Sac*I |
| 3 | CCG **GGT ACC** ACA AGC AGC TGA TGA AAA GCC A (SEQ ID NO:3) | *PKC1* flanking region ($F_1F_2$) forward primer | *Kpn*I |
| 4 | ACGC **GTC GAC** AAC CTG GAG TGG GAC AAA CAT (SEQ ID NO:4) | *PKC1* flanking region ($F_1F_2$) reverse primer | *Sal*I |
| 5 | CAT **GCATGC** TAA TAT AGG AAG CAT TTA ATA (SEQ ID NO: 5) | *TRP1* forward primer | *Sph*I |
| 6 | ACGC **GTCGAC** GCA AGT GCA CAA ACA ATA CTT (SEQ ID NO:6) | *TRP1* reverse primer | *Sal*I |
| 7 | ATT ATT CTC CAT GCG AGC CAG G (SEQ ID NO:7) | *$F_1F_2$-TRP1*-p*MET3*-*PKC1* integration check primer | |
| 8 | CAT GCT GCC TAT GTT GCA (SEQ ID NO:8) | *pMET3-SRB1-LEU2* integration check primer | |
| 9 | ACG ACA GAG AGA GAC CCA AG (SEQ ID NO:9) | p*MET3* reverse primer | |
| 10 | TGT CGA TTG GTG GGC ATT TGG G (SEQ ID NO:10) | *PKC1* diagnostic primer 1 | |
| 11 | CGT CAT GAA CTC TCG CGG ATT TGA TA (SEQ ID NO:11) | *PKC1* diagnostic primer 2 | |
| 12 | CGC GGA TCC AGC CAC AAG ACA AGC TAC AAC (SEQ ID NO:12) | *SRB1* forward primer | *BamH1* |
| 13 | ACT AGC ATG CAA TAC TAC AGA CAT TGA TAG CCA A (SEQ ID NO:13) | *SRB1* reverse primer | *Sph1* |

Table 3—Plasmids

| Plasmid | Description | Ref |
|---|---|---|
| pHAM8 | pUC19 derivative containing *MET3* gene | Mountain *et al.*, 1991 Yeast 7: 781 - 803 |
| pRS314 | pBLUESCRIPT-based *S.cerevisiae/E.coli* shuttle vector containing *TRP1* and an *ARS4-CEN6* fragment | Sikorski & Heiter (1989) Genetics 122:19-27 |
| pRS316 | pBLUESCRIPT-based *S.cerevisiae/E.coli* shuttle vector containing *URA3* and an *ARS4-CEN6* fragment | Sikorski & Heiter (1989) Genetics 122:19-27 |
| YEplac195-*PKC1* | YEplac195 derivative containing a 4.5 kb *PKC1* fragment at the unique *Sph*I site | Accession No. X75459 or L26355 |
| SRB1.9e | SRB1.9d derivative containing *pMET3-PSA1* cassette instead of *PSA1* and its homologous promoter | see Example 1 |
| pRS316-p*MET3* | pRS316 derivative containing *MET3* promoter | see Example 1 |
| pRS316-p*MET3-PKC1* | pRS316 derivative containing *MET3* promoter and *PKC1* coding sequence | see Example 1 |
| pRS316-$F_1F_2$-p*MET3-PKC1* | pRS316 derivative containing *PKC1* upstream region, *MET3* promoter and *PKC1* | see Example 1 |
| pRS316-$F_1F_2$-*TRP1*-p*MET3-PKC1* | pRS316 derivative containing *PKC1* upstream region, *TRP1*, *MET3* promoter and *PKC1* | see Example 1 |
| Pope-2μ | pFAKanMx4-based vector containing a 2μ replicator and *GFP* under the control of *RAD54* promoter | Walmsley *et al.* (1997) Yeast 13: 15 -35 |
| pUC19 | *E. coli* vector (Accession No M77789) | Yamish-Perron *et al.* (1985) Gene 33:103-119 |
| YCp50 | CEN-ARS-URA3 shuttle vector | Rose *et al.* (1987) Gene 60:237-243 |
| YEp13 | 2μ LEU2 shuttle vector | Broach *et al.* (1979) Gene 8:121-133 |
| YEplac195 | | Accession No. X75459or J01374 |
| pSRB1-2 | 14.5 kb chromosome IV fragment in YCp50 | Gardner *et al.* (1996) Yeast 12:411-413 |
| pSRB1-9b | *ClaI-BamHI* fragment of pSRB1-2 in pRS316 | see Example 1 |
| pSRB1-9d | pSRB1-9b with LEU2 in *NsiI-NheI* sites | see Example 1 |

**1.1.5 Transformation.**

*Escherichia coli* strains were transformed by electroporation with a Gene Pulser Apparatus as recommended by the manufacturer (Bio-Rad). Yeast strains were transformed either by a modification of the lithium procedure of Ito *et al.* (*J. Bacteriol.* **153:** 163-168, 1983), developed by Philipova (1985 Ph.D. Thesis, Institute of Molecular Biology, Bulgarian Academy of Science, Sofia, Bulgaria) and described by Stateva *et al.* (*Mol. Cell. Biol.* **11:** 4235-4243, 1991), or according to that of Hill *et al.* (*Yeast* **2:** 163-167, 1991).

**1.1.6 Lysis test and viability test**

Osmotic lysis tests were performed as described by Stateva *et al.* (*supra*) except that viability assays were not carried out in this study. Lysis data were normalised as the number of A260 units released per A600 unit of pre-lysis cell suspension.

For the viability tests, cells were counted using a haemacytometer, diluted, and then plated onto appropriately supplemented solid media. Viability is represented as the number of cells which grow on plates (multiplied by any dilution factor), calculated as percentage of the total number of counted cells.

**1.1.7 Methionine regulation of the p*MET3* expression cassettes**

Cultures of strains containing p*MET3*-regulated cassettes were grown in permissive growth medium until early exponential phase (OD600nm = 0.05), at which point they were split into two halves. To one half of each culture, methionine was added to a final concentration of 2mM (to form the modified growth media); to the other half, an equivalent volume of distilled water was added (controls). Cells were grown for additional periods of time for different purposes.

## CONSTRUCTION OF RECOMBINANT DNA MOLECULES:

### 1.1.8 Construction of pRS316-p*MET3*

pRS316-p*MET3* is a pRS316-based plasmid which contains the *MET3* promoter. The plasmid pHAM8 (Mountain *et al.*, 1991 Yeast 7: 781 - 803) was used as a source of the *MET3* promoter. It was digested with *Hin*dIII and *Eco*RV to release the p*MET3*-containing fragment (corresponding to the BgIII- EcoRV fragment of Yeast genome Accession No. X06413) which, after gel purification, was cloned into pRS316 (Sikorski & Heiter (1989) Genetics 122:19-27) that had been restricted with *Hin*dIII and *Sma*I. The resultant plasmid was named pRS316-p*MET3*.

### 1.1.9 Construction of pRS316-p*MET3-PKC1*

DNA coding for *PKC1* (Yeast genome accession number M32491) was obtained from a PCR using oligos 1 and 2 (Table 2) as primers and YEp195lac:*PKC1* (Yep195lac of Accession No. X75459 or L26355 containing PKC1 DNA of Accession No. M32491) of as the template. The DNA was restricted with *Not*I and *Sac*I. purified and cloned into pRS316-p*MET3* between the *Not*I and *Sac*I sites. The resultant plasmid was named pRS316-p*MET3-PKC1*.

### 1.1.10 Transformation of ZO124 with pRS316-p*MET3-PKC1*

pRS316-p*MET3-PKC1*, was transformed into a *pkc1*::*LEU2* yeast strain (ZO124, Table 1).

### 1.1.11 Construction of pRS316-$F_1F_2$-p*MET3-PKC1* and pRS316-$F_1F_2$-TRP1-p*MET3-PKC1*

To integrate the p*MET3-PKC1* regulation cassette at the homologous *PKC1* locus, a series of plasmids was constructed based on pRS316-p*MET3-PKC1* (Fig. 1). First, the *PKC1* upstream flanking region (which we designate $F_1F_2$) was inserted between the *Kpn*I and *Sph*I sites. $F_1F_2$ has the following DNA sequence:

ACAAGCAGCTGATGAAAAGCCAAGACATAAGTATTGTTGCCCACACT
GTGGGTCTTCATTTCCAAGATGTGCCATATGTCTCATGCCTCTAGGAA

CGTCAAACTTACCTTTTGTAATAAATGGGACGCAATCACGCGATCAAT
GCAGACAGAAGACTCTCAAGATGGTGCAAATCGCGAACTCGTAAGTA
GAAAACTGAAGTTGAACGAGTGGTTCAGCTTCTGTTTGAGTTGCAACCA
TGGTATGCATGCCGGTCACGCTGAAGAATGGTTTGACAGACATAATGTT
TGTCCCACTCCAGGTT

(SEQ I.D. NO. 14)

Second, a *TRP1* marker (a DNA molecule corresponding to Yeast genome accession No. V01341 or J01374) was cloned into pRS316-$F_1F_2$-p*MET3-PKC1* between the *Sph*I and *Sal*I sites. Finally, the construct pRS316-$F_1F_2$-*TRP1*-p*MET3-PKC1* was digested with *Kpn*I and *Sac*I and the fragment containing $F_1F_2$-*TRP1*-p*MET3-PKC1* was used to transform a *pkc1::LEU2* (ZO124, Table 1) host yeast strain. Transformants, which could grow up on medium without sorbitol and could not grow without leucine, were checked for correct integration by diagnostic PCR using two primer oligonucleotides (Oligos 5 and 6 in Table 2), one annealing to the *TRP1* marker and the other to sequence just outside the $F_1F_2$ fragment. This should yield a 1.4kb fragment and this was the case with transformant ZO126 (data not shown).

**1.1.12 Construction of pSRB1.9e**

pSRB1.9e was formed by making the following genetic manipulations of known DNA molecules/vectors:

**(a)** A *Cla*I-*Bam*HI fragment from pSRB1-2 (a Ycp50 recombinant plasmid comprising the *SRB1/PSA1* gene - Gardner *et al.* (1996) Yeast 12:411-413) was ligated into pRS316 Sikorski & Heiter *supra*) and the resulting construct named pSRB1.9b.

**(b)** The *LEU2* gene on a *Spe*I-*Nsi*I fragment isolated from Yep13 (described in Broach *et al.*, 1979) was then cloned into pSRB1.9b to form pSRB1.9d.

**(c)** The *SRB1/PSA1* coding sequence (without its promoter region) from pSRB1.9d was amplified in a PCR using the oligonucleotides of SEQ ID No.s 12 and 13 as forward and reverse primers respectively (see Table 2). The PCR product was treated with *Bam*HI and *Sph*I and subcloned into Ycp50 (described in Rose *et al.*, 1987) digested with the same enzymes to produce the plasmid YCp50-SRB1.

**(d)** A *Hind*III-*Bam*HI fragment (comprising the *SRB1* gene) from YCp50-SRB1 was then ligated with pMET3 on a *Hind*III-*Bam*HI fragment isolated from pRS316-pMET3 (see 1.1.8). The resultant construct was named YCp50-pMET3-SRB1.

**(e)** Finally, to allow integration into the *SRB1/PSA1* locus in yeast strains such as FY23, pSRB1-9e was made. Plasmid pSRB1-9d was digested with *Hind*III and *Nsi*I and ligated with the fragment comprising pMET3-SRB1 derived from digestion of YCp50-pMET3-SRB1 with the same restriction enzymes. The resultant plasmid pSRB1-9e may then be digested with *Apa*I and *Bst*1107I and the p*MET3-SRB1-LEU2* fragment obtained in this way used to transform FY23.

### 1.1.13 Integration of p*MET3-SRB1* in FY23

Integration of p*MET3-SRB1* into yeast chromosome IV was achieved by transforming, into *S.cerevisiae* strain FY23, with the *Apa*I/*Bst*1107I fragment of SRB1.9e containing *SRB1/PSA1* upstream and downstream flanking regions, surrounding the p*MET3- SRB1-LEU2*. The strain was named FY23-SRB1$^{MET3}$.

Transformants were selected by their inability to grow in the presence of 2mM methionine and were checked for correct integration using suitable diagnostic primers. The diagnostic PCR should result in a 1.1kb band (as was the case for FY23-SRB1$^{MET3}$.

### 1.1.14 Construction of strain ZO127, carrying both p*MET3-SRB1* and p*MET3-PKC1* integrated at their respective homologous loci

Plasmid SRB1.9e was digested with *Apa*I and *Bst*1107I and the fragment containing *SRB1/PSA1* upstream and downstream flanking regions, surrounding the p*MET3-SRB1-LEU2* cassette, was transformed into strain ZO126. Transformants which did not grow on medium containing 2mM methionine and 10% (w/v) sorbitol were further checked for correct integration at the *SRB1/PSA1* locus by diagnostic PCR as for FY23*SRB1*$^{MET3}$ (see Table 1). The resultant strain was named ZO127.

**1.1.15 Measurement of total protein release from cells**

Cells from 1-5 ml of culture grown at 30°C and 250rpm were washed and resuspended in 0.3ml breakage buffer (20mM Tris-Cl, 1mM EDTA, pH 7.5, 0.1M NaCl) containing protease inhibitor (Boehringer-Mannheim, cat. no. 1 836 153). The suspension was transferred to a 1.5ml microcentrifuge tube, to which 1ml of 100mg/ml zymolyase (ICN) was added. After incubation for 30 min. at 37°C, 0.3g of 0.45-0.6mm diameter glass beads (Sigma) were added. Cells were broken by vortexing four times at 4°C for 1 min., with an intervening period of 1 min. on ice between treatments. The cell debris and buffer were then pipetted into a fresh tube. The beads were washed with a further 0.2 ml of breakage buffer and the supernatants combined. The solution was cleared by centrifugation at 12,000g for 10 min. for total protein measurement.

The Bio-Rad protein assay was used since it is compatible with the amino acids contained in the minimal medium used. Protein samples were diluted to between 200µg and 1400µg/ml. When samples contained less than 25µg/ml of protein, the micro-assay was performed. Total protein was calculated by combining cell extract protein with the protein released into the medium. The protein released into the medium was then expressed as a percentage of the total protein.

**1.1.16 Measurement of fluorescence and GFP release from cells**

The plasmid Pope-2µ (Walmsley *et al.*, 1997 Yeast 13:1535-1545) containing jellyfish GFP under the control of the *RAD54* promoter was used to transform ZO127. 0.05% v/v methanesulfonic acid methyl ester (MMS, Sigma) was added to induce the expression GFP at very early exponential phase. For all GFP measurements, cells were grown at 25°C and 250rpm to avoid GFP misfolding. Cell extracts were isolated as above except that treatment with Zymolyase was carried out at 25°C for 90min. Fluorescence of the cell extract and lysate was determined using a 10nm excitation window at 488nm and a 5nm emission window at 511nm in a Perkin-Elmer luminescence spectrometer.

Since yeast proteins have a certain degree of fluorescence which means that it is impossible to differentiate homologous yeast proteins from GFP, ZO127 without GFP construct, grown under the same conditions, was included as the control. Brightness units for the control were defined as fluorescence divided by total protein in the cell extract and lysate (MII.3.2). We assumed that GFP constituted a negligible percentage of the protein in the cell extract or lysate of ZO127/Pope-2μ cells. So, fluorescence contributed by GFP in the cell extract of the sample was calculated as:

**Fc - Prot.c •Bc**

where Fc stands for fluorescence in sample cell extract; Prot.c denotes total protein in sample cell extract; Bc is the brightness units of the control cell extract.

The fluorescence contributed by GFP released into medium was calculated in a similar way as:

**Fm - Prot.m •Bm**

where Fm stands for fluorescence in sample medium (lysate); Prot.m denotes total protein in sample medium; Bm is the brightness units of the control medium. The yield of GFP released into the medium was obtained by dividing GFP in the medium by total GFP (GFP in the cell extract plus GFP in the medium).

## 1.2 RESULTS

### 1.2.1 Repression of *SRB1* leads to gradual cell lysis and release of cellular protein

The use of the regulation cassette p*MET3-SRB1* to determine the terminal phenotype of this essential gene has been studied by integrating the cassette at the *SRB1/PSA1* chromosomal locus in a standard laboratory strain, FY23. The expression of *SRB1/PSA1* was regulated by growing cells in minimal medium without methionine until the early exponential phase, at which point methionine was added. Cell viability, and total protein released to the medium, were monitored and compared with the Met$^-$ control. The results presented in Fig. 2 show that a significant proportion of the cells lost viability and released intracellular protein into the medium

upon addition of methionine. These results strongly suggest that cells lose their integrity and lyse gradually after switching off the expression of *SRB1/PSA1*.

**1.2.2 Repression of *PKC1* results in intensive cell lysis**

In order to test the applicability of the p*MET3* regulation of *PKC1*, the recombinant plasmid pRS316-p*MET3-PKC1* was transformed into a *pkc1*::*LEU2* host (ZO124; Table 1). The transformants were able to grow without sorbitol in the absence of methionine, but were unable to grow when 2mM methionine was added (data not shown). This indicated that p*MET3* could be used for the regulation of the expression of *PKC1*. The strain ZO126, which carries a p*MET3*-regulated *PKC1* gene, integrated at the homologous genomic site, was used in further experiments.

Strain ZO126 was grown on minimal medium until early exponential phase and subjected to the standard procedure of methionine regulation as described in Materials and Methods. Cell viability and total protein release were monitored. The results (Fig. 3) demonstrate that, upon addition of methionine, cells grew another 1-2 generations and then started to lose viability very quickly. At the same time, yeast proteins were released in to the medium. After 48 h. of repression, some 45% of intracellular protein was detected in the growth medium, indicating extensive cell lysis. Remarkably, after 3-4 generations under repressive conditions, cell viability started to increase, followed by a gradual decrease. This indicates that some of the cells survived the initial shock and were able to grow for some time, perhaps aided by the lysis products of the dead cells, but subsequently succumbed to lysis.

**1.2.3 Concomitant repression of *SRB1/PSA1* and *PKC1* leads rapidly to extensive cell lysis**

Cells of strain ZO127, in which integrated copies of both *SRB1/PSA1* and *PKC1* are expressed from the *MET3* promoter, were grown in minimal medium until early exponential phase, methionine was then added to repress this expression. The results (Fig. 4) show that, upon addition of methionine, cells lost viability more

quickly than those having either of the single p*MET3* expression cassettes (compare these results with those in Figs 2 and 3). Within 24 h, more than 80% of the cells lysed, releasing the bulk of their intracellular protein into the growth medium. These results indicate that the lysis phenotype conferred by the repression of *SRB1/PSA1* and *PKC1* is additive. Cells in which both *SRB1/PSA1* and *PKC1* expression may be regulated are particularly suited for use in the biotechnology industry in applications where inducable lysis is required

### 1.2.4 *SRB1/PSA1* and *PKC1*-repressed cells can release an heterologous protein (GFP) from its intracellular location

Green Fluorescent Protein (GFP), a heterologous protein, was used to demonstrate the applicability of cells in which repression of *SRB1/PSA1* and *PKC1* may be induced. For this purpose, the plasmid Pope-2μ (Walmsley *et al.*, 1997 Yeast 13:1535-1545) was transformed into ZO127 and the transformants grown in selective medium. This 2μ-based plasmid expresses the coding sequence of jellyfish green fluorescent protein from the *RAD54* promoter (Walmsley *et al.*, 1997 *supra*). A methionine repression experiment was performed and GFP release measured. Little GFP was released into the medium by cells in the control ($Met^-$) culture. However, more than 30% of cellular GFP was found in the medium of the experimental ($Met^+$) culture, in which the expression of both *SRB1/PSA1* and *PKC1* was repressed for 48 h. (Fig. 5). This demonstrates that this lysis system is able to release both homologous and heterologous proteins from yeast cells.

## 1.3 DISCUSSION

Compared with other cell lysis systems, where a temperature shift or the removal of an osmotic stabiliser is required, cells according to the first aspect of the invention may be induced to lyse according to the method of the second aspect of the invention by the addition of a simple chemical to repress the expression of two genes (*SRB1/PSA1* and *PKC1*) involved in wall biogenesis to produce spontaneous cell lysis. Although the concentration of methionine used experimentally to repress the

*MET3* promoter was 2mM, we have found it can be altered to achieve the same repressive effect (e.g. it may be lowered to 0.05-1.0mM). A methionine analogue or cheaper metabolites in the methionine/threonine pathway may also be used as repressive agents for an industrial-scale process. An initial experiment has indicated that 0.2% yeast extract could suppress the cell growth of ZO125 (p*MET3-SRB1*) as well as 2mM methionine, whereas 2% yeast extract could not suppress the cell growth of ZO126 (p*MET3-PKC1*) at all (data not shown).

## EXAMPLE 2

During the studies performed in Example 1 it was surprisingly noticed that the new pattern of expression of *SRB1/PSA1* and/or *PKC1* from p*MET3* resulted in changes in the flocculation phenotype of transformed cells. Further investigations were therefore performed which confirmed that such cells were useful according to the method of the third aspect of the present invention.

### 2.1. Materials and Methods

#### 2.1.1 Strains and media

The *Saccharomyces cerevisiae* strains used in this study, together with their genotypes and sources, are listed in Table 1 in Example 1 above. The strains were grown in minimal medium (SD) prepared according to Kaiser *et al.* (*supra*). Nutritional supplements were added to the medium as necessary.

#### 2.1.2 Plasmids and transformation

See Example 1.

#### 2.1.3 Northern analysis

Yeast total RNA was extracted and separated on a 1.5% (w/v) denaturing agarose gel according to the protocol described by Kaiser *et al.* (*supra*). Following blotting onto a nylon membrane (positively charged; Boehringer Mannheim), it was

hybridised with the appropriate DNA probe labelled with $\alpha$-$^{32}$P-dCTP using a *Rediprime*$^{TM}$ kit (Amersham, UK). Prehybridisation, hybridisation, and washing steps were carried out following the procedure described by Engler-Blum *et al.* (Anal Biochem 210: p235-244, 1993). After washing, the membrane was wrapped in *Saran-Wrap* and exposed to a BioRad imaging screen (type BI) which was then developed with the BioRad phosphorimager (GS-363) and analysed with Molecular Analyser$^{TM}$ software.

### 2.1.4 Methionine regulation of p*MET3* expression cassettes

Cultures of strains containing p*MET3*-regulated cassettes were grown according to method 1.1.7.

### 2.1.5 Construction of isogenic wild-type strain, ZO123

Used as the control in this study, a isogenic wild-type strain was constructed by transforming a PCR product containing the *PKC1* gene into strain ZO124. The *PKC1* coding sequence was PCR-amplified using YEplac195-*PKC1* (see Example 1) as the template, a forward primer of sequence ID NO 1 and a reverse primer of sequence ID NO 2. Those transformants which could grow without 10% (w/v) sorbitol, but could not grow without a leucine supplement, were further checked for correct integration. This was done by diagnostic PCR, using one oligonucleotide of sequence ID NO 10, which anneals to *PKC1* but not the deletant sequence and another of sequence ID NO 11, which anneals outside the *PKC1* fragment. This gives a 0.85kb fragment, as is the case with ZO123 (data not shown).

### 2.1.6 Construction of ZO125

ZO125 is based on ZO123 with p*MET3-SRB1-LEU2* integrated at the chromosomal *SRB1/PSA1* locus. SRB1.9e was digested with *Apa*I and *Bst*1107I. The fragment containing the *SRB1/PSA1* upstream region, p*MET3-SRB1-LEU2* and *SRB1/PSA1* downstream region was transformed into ZO123. The correct integration

was confirmed both phenotypically and by diagnostic PCR as previously used for FY23*PSA1*$^{MET3}$ (Table 1).

### 2.1.7 Flocculation measurements

A cell culture (100ml) was grown in SD medium containing 2% (w/v) glucose for 48h. until stationary phase had been reached. A portion (50ml) of this culture was placed in a 50ml Falcon tube. Cells were collected by centrifugation at 5000rpm for 5 min. The medium was poured away and cells were washed once with 250mM EDTA and twice with sterile water before resuspension in water to a final concentration of 1-4 x $10^9$ cells/ml. An aliquot (1ml) of this suspension was used for quantification of flocculation as described by Stratford & Assinder (Yeast 7: 559-574, 1991).

## 2.2 RESULTS AND DISCUSSION

### 2.2.1 p*MET3* regulation of *PKC1* causes flocculation under conditions of methionine repression

Strain ZO126 (Table 1), carrying the p*MET3-PKC1* cassette, was grown until early exponential phase when methionine (2mM) was added to half of the culture in order to repress the expression of *PKC1*. Incubation was then continued at 30°C for a further 48h. The other half of the culture was grown for the same time in the absence of methionine. It was observed that flocs were formed in the stationary culture grown under repression condition (Fig. 6). Cells were harvested and flocculation was quantified as described in Materials and Methods. At the same time, the wild-type strain ZO123, grown under similar conditions, with and without methionine, was included as a control. The results (Fig. 7) show that the wild-type cells have very weak flocculation ability, irrespective of whether methionine was present or not. The flocculation ability of the strain in which *PKC1* is under the control of p*MET3*, was about 50% higher than that of the wild type grown in medium without methionine. However, this strain showed at least a three to four times greater ability to flocculate when methionine was added to repress the expression *PKC1*.

Although we do not wish to be bound by any hypothesis, we believe repression of *PKC1* can induce flocculation because it has been shown to be involved in glucan biosynthesis. Electron micrographs of the cell wall of a temperature-sensitive *pkc1* mutant show that the wall loses its normal organisation at the restrictive temperature. At the permissive temperature, the wall consists of an inner transparent layer and an outer, dark-stained, mannoprotein layer. In contrast, at the restrictive temperature, the transparent layer almost completely disappears and the entire wall stains dark, suggesting that the inner (skeletal) layer of the wall has been affected. Thus upon addition of methionine to a ZO126 culture, cells are depleted of Pkc1p. We believe this is results in much thinner inner cell walls which render the mannose side-chains in the incorporated mannoprotein more accessible to flocculin and thereby lead to a higher ability for flocculation.

### 2.2.2 Repression of *SRB1/PSA1* under the control of the *MET3* promoter causes flocculation

*SRB1/PSA1* repression caused flocculation of ZO125. A flocculation test was performed on ZO125 cells which had been grown in permissive conditions until early exponential phase and then cultured for another 48 h in the presence of 2mM methionine. The wild-type strain, ZO123, grown under the same conditions, was used as the control. As shown in Fig. 9, repression of *SRB1/PSA1* in ZO125 leads to a 5-fold increase in flocculation.

Srb1p/Psa1p activity results in the production of GDP-mannose, an activated sugar that is involved, either directly or via the dolichol pathway, in all kinds of protein glycosylation, including GPI anchor synthesis. The product of the dominant flocculation determinant, *FLO1*, has been shown to be a GPI-anchored cell surface protein. Thus, it is conceivable that repression of *SRB1/PSA1* results in a failure to incorporate Flo1p into the cell wall, or in its failure to function properly if incorporated. Mutants, like *mnn9*, that lack the outer polymannose chain on their mannoproteins grow slowly and display a clumpy morphology. Thus, the flocculation

resulting from *SRB1/PSA1* repression could be due to a profound change in cell-surface characteristics, including hydrophobicity.

### 2.2.3 Constitutive expression of *SRB1/PSA1* under the control of the *MET3* promoter causes flocculation at all stages of the batch growth cycle

The growth of strain ZO125, harbouring the p*MET3-SRB1* cassette, in SD medium under expression conditions (without methionine), was characterised by the following phenotypes. The culture was 'flaky' throughout batch growth. The flocculated cells at the exponential phase are shown in Fig. 8. This suggests that the expression of *SRB1/PSA1* from p*MET3*, unlike that of *PKC1* (above), leads to flocculation at any growth phase. ZO125 cells grew faster, with a doubling time of 1.9h. (while the doubling time of the wild-type strain ZO123 is 2.1h.) and appeared to have bigger cell volumes (Fig. 8), in comparison to the wild-type isogenic control, ZO123. When the cells from both strains were grown to stationary phase and subjected to the flocculation test, it was observed (Fig. 9) that the cells carrying the p*MET3-SRB1* expression cassette had a five times higher ability for flocculation than the control strain.

We also found that cells transformed with the p*MET3-SRB1* cassette had a flocculating phenotype when grown in "permissive" media (i.e in the absence of methionine) (see Figs 8 and 9). This suggested to us that constitutive expression of *SRB1/PSA1* under the regulation of *pMET3* causes flocculation.

We further investigated how *SRB1/PSA* expression from the *MET3* promoter caused flocculation by investigating whether *pMET3-SRB1* led to flocculation in a wild-type strain. The plasmid SRB1.9e, which carries the p*MET3-SRB1* cassette in a centromeric vector. was transformed into the wild-type strain ZO123, which has a wild-type copy of *SRB1/PSA1* under the control of its own promoter. The transformants were grown in the same medium as that for ZO125, the strain containing an integrated copy of p*MET3-SRB1* at the *SRB1/PSA1* chromosomal locus.

A flocculation phenotype, the same as that of ZO125, was observed in all growth phases and the cell volume appeared larger compared with that of the wild type (Fig. 8). The flocculation test, which was performed after the cells had been grown to stationary phase, showed that this transformant flocculates to the same degree as ZO125. This result confirmed that the flocculation phenotype caused when *SRB1/PSA1* is transcribed from p*MET3* is not due to the gene's underexpression but, rather, is the result of its constitutive expression.

The result of the viability test on FY23*PSA1*$^{MET3}$ shows that cell viability is not affected by the constitutive expression of *SRB1/PSA1*, suggesting that sufficient Srb1/Psa1p is synthesised under the control of p*MET3* to allow yeast to go through its cell cycle. However, when *SRB1/PSA1* is expressed from its own promoter, its transcription level increases some 4- to 6-fold at START. Thus the constitutive expression of *SRB1/PSA1* from p*MET3* would hyperactivate glycosylation at all other cell cycle phases; it may well be this that leads to enhanced cell growth and flocculation.

We have therefore established that the interaction between *SRB1/PSA1* expression and the dominant flocculation genes is important in determining a flocculant phenotype and the repression of *SRB1/PSA1* expression or the alteration of wildtype *SRB1/PSA1* expression patterns (e.g. constitutive expression under the *pMET* promoter) can induce flocculation. Thus genetically modified yeasts in which *SRB1/PSA1* expression is modulated may be used industrially according to the method of the fourth aspect of the invention to induce flocculation.

Flocculation provides a means of efficient separation of cells from the medium. However, most cell wall mutants with enhanced flocculation capacity are affected in cell growth or cell viability, like *KRE6* and *mnn9*. The flocculation resulting from *SRB1/PSA1* expression from the *MET3* promoter has no overt effect on

cell growth and viability. Thus cells in which *SRB1* is under the regulation of an inducable promoter may be used industrially when flocculation only is required.

### 2.2.4 Flocculation ability of a double (p*MET3-PSA1*, p*MET3-PKC1*) mutant

The strain ZO127 (see Table 1), carrying both the p*MET3-PSA1* and p*MET3-PKC1* cassettes, was used to test flocculation under non-repressing ($Met^-$) and repressing ($Met^+$) conditions. Under both conditions, cells from this strain formed very large flocs (Fig. 10) at all growth phases. After growth for 48h, cells were harvested and subjected to a flocculation test (Fig. 11). Around 95% of the cells, from either the $Met^-$ or the $Met^+$ culture, formed large aggregates. This indicates that ZO127 cells have a higher ability for flocculation than those bearing the single cassettes (compare Figs. 7, 9, and 11), suggesting that the flocculation effects resulting from p*MET3*-regulated *PKC1* and *SRB1/PSA1* expression or repression are additive.

Thus we have established that modulation of *SRB1/PSA1* alone (see 2.2.3) or *PKC1* and *SRB1/PSA1* (2.2.4) is directly linked to flocculation. These results suggest that morphogenes in yeast must obey cell cycle controls whilst responding to environmental changes.

Concomitant repression of *SRB1/PSA1* and *PKC1* leads to extensive cell lysis and flocculation, making the system useful in heterologous protein expression and downstream operations in which controlled cell lysis and efficient separation of cell ghosts from the medium can be realised at the same time.